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[11] Expression of Genes in Yeast Using the *ADC1* Promoter

By GUSTAV AMMERER

Compared to higher eukaryotic cells, the yeast *Saccharomyces cerevisiae* seems to recognize slightly different signals for gene expression. Therefore it was desirable to develop a vector system capable of promoting high levels of transcription for any coding sequence introduced into yeast cells. Such vectors should be suitable for studying the production of foreign proteins in yeast. They also may be used to enhance the synthesis of yeast gene products, for example, regulatory proteins, which are normally present only at low levels. In this context it is also possible to study the physiological effects of constitutive expression of otherwise highly regulated genes. Because yeast alcohol dehydrogenases and their genes have been well characterized,[1] we chose to use the 5′-flanking sequence of the *ADC1* gene (coding for *ADH1*) as a portable promoter. Although the *ADC1* gene probably does not contain the most powerful *polII* promoter in yeast, the relative abundance of this glycolytic enzyme is reflected on transcriptional level. *ADH1* mRNA is estimated to be 1–2% of poly(A) RNA.

Bennetzen sequenced about 2100 nucleotides of the *ADC1* region including 750 base pairs (bp) flanking the 5′ end and 320 bp flanking the 3′ end.[2] A presumptive Goldberg–Hogness box can be found at position −128 from the initiator codon. The 5′ ends of the mature mRNA have been mapped around nucleotides −37 and −27. These presumptive transcription initiations sites are preserved when the gene is maintained on a plasmid. As with other highly expressed yeast genes, the untranslated leader region of the mRNA is almost completely devoid of G residues. This strong bias is probably not essential for transcription initiation but might be critical for the translational capacity of the mRNAs. In order to ensure high translation rates for hybrid mRNAs containing non-*ADH1* coding information, it would be preferable to leave the untranslated leader of *ADC1* intact. By joining the different genes within the translated regions one normally generates a fusion protein, which might be unacceptable for many purposes. The cleanest approach would be to replace the

[1] T. Young, V. Williamson, A. Taguchi, M. Smith, A. Sledziewski, D. Russell, J. Osterman, C. Denis, D. Cox, and D. Beier, *in* "Genetic Engineering of Microorganisms for Chemicals," (A. Hollaender, D. Demoss, S. Kaplan, J. Konisky, D. Savage, and R. Wolfe, eds.), p. 335. Plenum, New York, 1982.

[2] J. Bennetzen and B. D. Hall, *J. Biol. Chem.* **257**, 3018 (1982).

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coding region of *ADC1* directly at the initiator *ATG*. This could be accomplished, for example, by the lengthy procedure of chemically synthesizing an oligonucleotide "bridge" fragment to reach from a restriction site 5′ to *ATG* in *ADC1* to a site 3′ to *ATG* in the coding region to be expressed. As a more rapid means of joining the *ADC1* 5′-flanking region to a variety of genes, I introduced convenient restriction endonuclease sites proximal to the initiator *ATG* of the yeast *ADC1* gene. A 1600-bp *Sau*3A fragment served as starting material and was subcloned after *Bam*HI linkers (5′) and *Hin*dIII linkers (3′) had been attached. This piece contained 108 bp of translated *ADC1* sequence and 1500 bp of the 5′-flanking sequence. It was trimmed further on its 3′ end by either a combination of ExoIII and S1 nucleases or later by *Bal*31 nuclease, which proved to be preferable over the first method. The pool of fragments shortened to the desired size was ligated to different molecular linkers, recut with the specific restriction endonucleases, purified by electrophoresis, and ligated into appropriate vectors. After transformation into *E. coli*, individual colonies were screened for inserts, and the position of the linker was determined.

Construction of Promoter Fragments

All enzymes were obtained from Bethesda Research Laboratories (BRL) and normally used as recommended by the supplier. Molecular linkers were purchased from Collaborative Research or BRL.

Step 1. Bal31 Nuclease Digestions. When *Bal*31 nuclease was used in our experiments we started with a promoter fragment that was already shortened and subcloned after a previous *Exo*III–S1 nuclease treatment and had a linker at position +28. Ten micrograms of linearized DNA (about 5 pmol of DNA ends) were dissolved in H_2O and brought to a final concentration of 20 m*M* Tris-HCl, pH 8.1, 200 m*M* NaCl, 12 m*M* $MgCl_2$, 1 m*M* EDTA, and 100 μg of bovine serum albumin (BSA) per milliliter in a volume of 200 μl. The prewarmed sample was digested with 0.5 unit of *Bal*31 enzyme at 30°. After 15 and 30 sec, 100-μl aliquots were mixed with stop solution (100 μl of phenol–chloroform–isoamyl alcohol, 50:50:1 + 5 μl of 250 m*M* EDTA). Under these conditions we found that the enzyme removed 1–2 bp per second per DNA end. To get an even distribution of fragments digested to different length, the reaction mixture was sometimes slowly dripped into the stop solution, using prewarmed pipette tips. After ether extraction and ethanol precipitation of the aqueous phase, the DNA was dissolved in 50 μl of H_2O. Two-microliter aliquots were cut with a convenient restriction enzyme (*Bam*HI or *Sph*I), and the amount of degradation was determined by separating the fragments on agarose gels. Digestions with *Bal*31 nuclease turned out to be

quite variable depending on the DNA preparation, DNA concentration, or the preparation and storage of the enzyme. Therefore it is useful to optimize incubation conditions using preparative amounts of DNA.

Step 2. Linker Addition. Although *Bal*31 nuclease should leave a large fraction of DNA molecules with blunt ends, we found that ligation efficiency was much higher after incubation with DNA polymerase I Klenow fragment. Ten microliters of *Bal*31-treated DNA solution (~1 μg) was brought to a final volume of 40 μl in 10 m*M* Tris-HCl, pH 7.4, 6 m*M* $MgCl_2$, 2 m*M* 2-mercaptoethanol, 23 μM concentration of each deoxyribonucleotide, and 100 μg of BSA per milliliter. The sample was incubated with 2 units of DNA polymerase I Klenow fragment for 30 min at room temperature. The reaction was stopped by heating for 10 min at 65°. At the same time molecular linkers were phosphorylated in a 40-μl volume of 10 m*M* Tris-HCl, pH 7.6, 10 m*M* $MgCl_2$, 1 m*M* spermidine, 10 m*M* DTT, and 100 μg of BSA per milliliter with 30 μCi of labeled [^{32}P]ATP and 10 units of T4 polynucleotide kinase. After 10 min at 37°, 1 μl of 20 m*M* ATP was added and the incubation was continued for 30 more minutes. The reaction was stopped by heating for 10 min at 65°. Forty microliters of blunt-end DNA was combined with 20 μl of phosphorylated linkers and 1 μl of 20 m*M* ATP, and 2.5–3 units of T4 ligase were added. The sample was kept at 14° for more than 12 hr. The T4 ligase was inactivated by heat (10 min, 65°). Sodium chloride was added to a final concentration between 60 m*M* (*Hin*dIII linkers) and 150 m*M* (*Xho*I linkers). The sample was recut with an excess of the enzyme specific for the linker (100 units) and the normal amount of enzyme cutting upstream from the promoter (*Bam*HI, 5 units) for 2–3 hr. The reaction was terminated by heating to 65°. Small aliquots were taken before and after digestion with the restriction enzymes and separated on a 8% nondenaturing acrylamide gel and autoradiographed for several hours. In this way kinasing of the linkers, efficient ligation, and complete recutting could be assured.[3] Fragments were purified and separated from the molecular linkers by electrophoresis on agarose or acrylamide gels. The DNA was visualized by staining with ethidium bromide, and the fragments were electroeluted and ligated into plasmids with the appropriate restriction endonuclease sites.

Step 3. Screening for Position of the Molecular Linker. Minipreparations of plasmid DNA were isolated from 5 ml of L-broth culture using the method described by Birnboim and Doly.[4] The DNA was dissolved in 100 μl of H_2O. Five microliters were cut with the restriction enzyme corresponding to the linker in 20-μl reaction volumes. After digestion, the

[3] H. M. Goodman and R. J. MacDonald, this series, Vol. 68, p. 75.
[4] A. C. Birnboim and J. Doly, *Nucleic Acids Res.* 7, 1513 (1979).

sample was heated for 10 min at 65°. DNA polymerase I Klenow fragment (0.4 unit) and 0.5 μCi of the suitable ^{32}P-labeled deoxyribonucleotide were added and incubated for 30–40 min at room temperature. The reaction was terminated again by heat. The DNA was then recut with *Alu*I, generating small, labeled fragments. The size of these fragments was dependent on the distance from the linker to the *Alu*I site at position −36. After ethanol precipitation, the pellets were dissolved in formamide dye mixture and analyzed on a 20% sequencing gel. Chemical sequencing reactions from a fragment of known size were used as size markers.[5] In this way the position of the linker could normally be calculated with an error of plus or minus one nucleotide.

Principally, the screening for a functional yeast promoter can be facilitated by restoring promotion and function of a selectable or easily detectable gene product in yeast, e.g., β-galactosidase,[6] cytochrome *c*, enzymes of the adenine pathway, or galactokinase.

Vectors Containing the *ADC1* Promoter

By the method described above, a variety of promoter fragments were obtained and characterized. Vectors with *Bam*HI, *Hin*dIII, *Xho*I, and *Eco*RI linkers inserted into the untranslated leader region are available (Figs. 1A and 1B). These vectors are related either to *ars1*-containing vectors such as YRp7[7] or 2 μm DNA vectors such as YEp13.[8] In all cases a functional promoter can be cut out as a 1500-bp fragment, using *Bam*HI

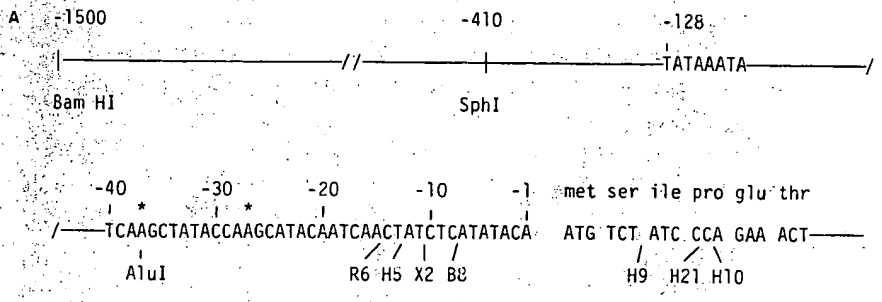

FIG. 1A. Sequence of the N-terminal region of the *ADC1* gene. the stars mark the 5′ ends of the mature *ADH1* mRNAs. The letters indicate the site of the linker attachment to the promoter: H = *Hin*dIII linker CCAAGCTTGG; B = *Bam*HI CCGGATCCGG; X = *Xho*I CCTCGAGG, R = *Eco*RI GGAATTCC.

[5] A. Maxam and W. Gilbert, this series, Vol. 65, p. 497.
[6] M. Rose and D. Botstein, this volume [42].
[7] A. Tschumper and J. Carbon, *Gene* **10**, 157 (1980).
[8] J. R. Broach, J. N. Strathern, and J. B. Hicks, *Gene* **8**, 121 (1979).

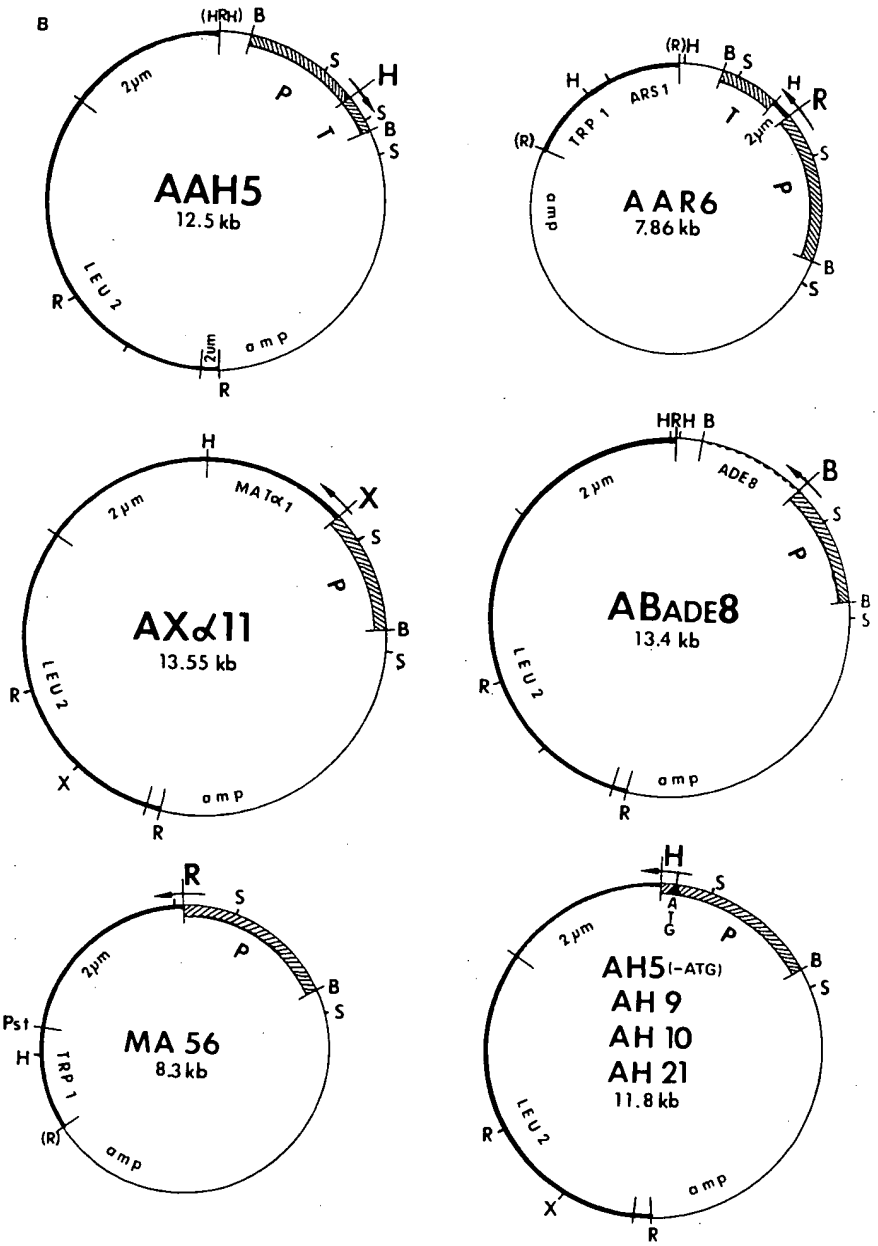

FIG. 1B. Vectors containing the *ADC1* promoter fragment. The promoter with the *Eco*RI linker at position −14 (R6) is used in plasmid pMA56 and in plasmid pAAR6. The fragment with the *Hin*dIII linker at position −12 (H5) is used in pAAH5 and pAH5. Fragments with a *Hin*dIII linker inserted at positions +7, +11, +12, are used in plasmids pAH9, pAH21,

and the enzyme specific for the linker, or as a ~410 bp fragment, using *Sph*I endonuclease. Plasmids pAH9, pAH10, and pAH21 retained the initiator methionine of the *ADC1* gene and result in the synthesis of a fusion protein when joined to a coding region. Each plasmid has a single *Hin*dIII site in a different reading frame. Vector pAH5 is similar to the previous plasmids except that the *ADC1* sequence is deleted to position −12 upstream from the *ATG*. In pMA56 the *ars1* sequence of YRp7 was replaced by the *Pst*I–*Eco*RI fragment containing the replication origin of yeast 2 μm DNA. The *Eco*RI site between the *TRP1* gene and the *amp*^{r} region is destroyed. The *ADC1* promoter was inserted as a *Bam*HI–*Eco*RI piece into the *tet*^{r} region. In respect to stability in yeast, the plasmid behaves similarly to YEp13. Five primed flanking sequences seem to be generally responsible for directing transcription to initiate at specific sites. As expected, we found in S1 nuclease experiments using a chimeric combination of *ADC1* with rat growth hormone cDNA[9] that the mRNA ends were unaltered for the hybrid gene. In addition to faithful transcription initiation, termination and poly(A) tailing of the RNA may be important for gene expression. In our experiments, the mammalian cDNA did not provide functional signals for termination in yeast. Instead, transcription initiating from the *ADC1* promoter fragments terminated within the 2 μm part of the vector. A 2 μm DNA fragment containing the replication origin (bp 105–1998 of the B form)[10] hybridizes specifically to a distinct poly(A) transcript starting from the *ADC1* promoter (Fig. 2). The transcript covers about 600 bp including the carboxy-terminal end of an open-reading frame located on the same DNA strand as the *ADC1* promoter.[11] This reading frame was assigned to the flipping enzyme (Able) encoded by 2 μm plasmid. It seems that this gene provides a signal for poly(A) addition and its RNA usually terminates within the long inverted repeat of 2 μm DNA.[10]

We also constructed vectors with the C-terminal and 3′-flanking region of the *ADC1* gene (450 bp *Hin*dIII–*Bam*HI fragment, referred to as ADH terminator) downstream from the promoter (Fig. 1B). These vectors

[9] P. H. Seeburg, J. Shine, J. Martial, J. D. Baxter, and H. M. Goodman, *Nature* (*London*) **270**, 486 (1977).
[10] J. R. Broach, J. F. Atkins, J. F. McGill, and L. Chow, *Cell* **16**, 827 (1979).
[11] J. L. Hartley and J. E. Donelson, *Nature* (*London*) **286**, 860 (1980).

pAH10, respectively. The plasmid pAXα11 contains the promoter fragment with an *Xho* linker at −10 (X2); the plasmid ABade8 contains the fragment with the *Bam*HI linker at position −7 (B8). P = *ADC*1 promoter, T = *ADC*1 terminator, X = *Xho*I, B = *Bam*HI, R = *Eco*RI, *H* = *Hin*dIII, S = *Sph*I. A letter set in parentheses means that a previous restriction endonuclease site has been destroyed. This was accomplished by cutting the site with the specific enzyme, filling in the recessive ends with DNA polymerase I Klenow fragment, and religating the blunt ends.

showed two to three times higher expression of an inserted human interferon gene when compared to a plasmid terminating in 2 μm DNA. Effects of the 3′-flanking sequence on the mRNA stability are possible but not proved. Plasmids pAAR6 and pAAH5 also offer the advantage that one can transfer from them to other vectors a single integral unit of promoter–inserted sequence–terminator as either *Bam*HI or *Sph*I fragment. Many of the common yeast–*E. coli* vectors contain a single *Bam*HI or *Sph*I site within the *tet*^{r} region of pBR322. In pAAR6, which contains a single *Eco*RI site for cloning, a small 2 μm *Eco*RI–*Hin*dIII (105 bp) fragment served as adapter between the terminator and promoter of *ADC*1. The vector was constructed from YRp7[7] after the two *Eco*RI sites had been eliminated. Plasmid pAAH5 provides a *Hin*dIII site for cloning. The short DNA sequence between the original two *Hin*dIII sites of YEp13 was deleted, and the sites were destroyed.

In the plasmid ABade8 a *Bam*HI–*Bam*HI promoter piece was placed in front of a truncated *Drosophila melanogaster ADE*8 gene.[12] This plasmid can complement *ade*8 mutations in yeast. Plasmid *AX*α11 (*Xho*I linker at position −10) was constructed by ligating a *Xho*I–*Hin*dIII fragment of the *MAT*α*1* gene[13] together with a *Bam*HI–*Xho*I promoter fragment into *Bam*HI, *Hin*dIII cut YEp13. All the vectors described can confer ampicillin resistance to *E. coli* cells. In yeast plasmids pAH5–pAH21, pAAH5 pABade8, and pAXα11 complement *leu2* mutations. Plasmid pMA56 and pAAR6 contain the *TRP1* gene as a selectable marker.

Regulation of ADHI

Because control of transcription plays a major role in regulating gene expression, one might anticipate that the joining of the 5′-flanking region of *ADC1* to other coding regions would impart *ADC1*-specific regulation on these genes. Yeast *ADH1* was originally considered to be a constitutively produced enzyme. Studies by Denis *et al.*[14] have shown that expression of the *ADC1* gene is regulated to some extent. When yeast cells are shifted from glucose to ethanol-containing medium, the amount of the enzyme, the level of its translatable mRNA, and the amount of RNA detectable by hybridization on Northern blots decreases considerably. A quite similar effect can be observed when cells reach late log or stationary growth phase. The drop in *ADH1* expression is normally accompanied by derepression of the isoenzyme *ADH11*.[1] At the same time a new relatively

[12] S. Henikoff, K. Tatchell, B. D. Hall, and K. A. Nasmyth, *Nature* (*London*) **289**, 33 (1981).
[13] K. Tatchell, K. Nasmyth, B. D. Hall, C. R. Astell, and M. Smith, *Cell* **27**, 26 (1981).
[14] C. Denis, J. Ferguson, and T. Young, *J. Biol. Chem.*, in press (1983).

weakly expressed transcript appears that hybridizes not only to *ADCI* coding region, but also specifically to the 5′-flanking sequence (Fig. 2). It starts about 1000–1100 nucleotides upstream from the start points originally mapped for *ADHI* message. Because of the upstream start and stop signals for translation, it should not be a translatable *ADHI* mRNA. Additionally it should not act as a precursor to *ADHI* mRNA. Beier and Young[15] have shown that deletions of the region upstream from the TATA box or upstream from the *Sph*I site do not abolish *ADHI* activity. Instead, in this case high expression of *ADHI* continues even when cells are grown on ethanol as carbon source. If more than 1400 bp of flanking sequence are present, *ADHI* activity in those cells is regulated in a similar way as in wild-type strains. In all these experiments the *ADCI* gene was maintained on a plasmid.

No systematic studies of transcriptional regulation have been done with fusions using the *ADCI* promoter. However, most of the data indicate that production of foreign proteins behaves similar to *ADHI* expression. For those cases in which yeast cultures are grown to high density, it might therefore be preferable to use a small promoter piece (e.g., between the linker and the *Sph*I). We also found that on a plasmid the expression of the larger transcript seemed considerably enhanced compared to the amount of the same RNA detected from chromosomal *ADCI*. One is tempted to speculate that read-through from the upstream promoter site is responsible for the low expression of *ADHI* in cells grown on ethanol. But generally speaking the significance of this read-through transcript to *ADHI* regulation, as well as the whole nature of the *ADCI* control mechanism, is still obscure.

Conclusion

The *in vivo* expression obtained by joining the yeast *ADCI* promoter to other yeast genes and to heterologous genes must be evaluated at two different levels. For all coding regions tested, attachment to the *ADCI* 5′ flanking sequence promoted active transcription in yeast, as evidenced by a strongly hybridizing band on Northern blots probed with the non-*ADH* coding sequence. However, at the level of stable protein product accumulated in yeast, the results have been much more variable, with high expression in the case of human α-interferon[16] and moderate or undetectable levels for other genes (hepatitis B surface antigen, bovine parathyroid

[15] D. Beier and T. Young, *Nature* (*London*) **300**, 724 (1982).
[16] R. A. Hitzeman, T. E. Hagie, H. L. Levine, D. V. Goeddel, G. Ammerer, and B. D. Hall, *Nature* (*London*) **293**, 717 (1981).

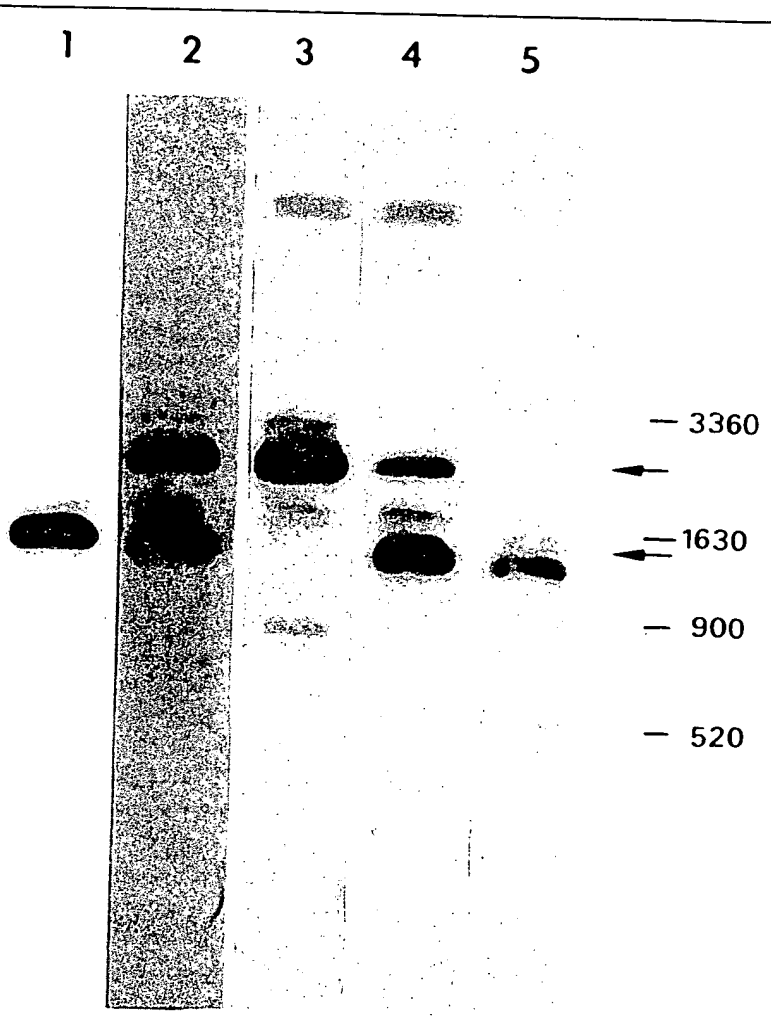

FIG. 2. Characterization of transcripts initiating from the *ADC1* promoter. Yeast strain PS23-6A (α *leu2 trp1*) was transformed with YEp13 or the vector pAHG23 related to pAH5 containing a ~800 bp fragment of rat growth hormone cDNA[9] inserted into the *Hin*dIII site. Glyoxylated samples of poly(A)-containing RNA (10 μg) were fractionated on a 1.2 agarose gel, transferred to nitrocellulose paper, and probed with different DNA fragments ^{32}P-labeled by nick translation (see P. S. Thomas, this series, Vol. 100 [18]). 3 to 5 × 10^5 cpm were used for hybridization. The autoradiograms were exposed with intensifying screen at

hormone, or rat growth hormone, respectively). These differences in expression from one case to another may result from a variety of posttranscriptional and posttranslational effects, e.g., mRNA stability, mRNA processing, translational capacity of the mRNA, stability of the protein, modification or processing of the protein, and more. Low expression results are also explainable by differences in the preferred codon usage between higher eukaryotes and *S. cerevisiae*.[17] But unfortunately no systematic experiments have been done so far to substantiate any of these explanations. Finally it should be mentioned that identical plasmids gave variable expression of a foreign gene in different yeast strains. This, however, is no surprise considering how much the expression of yeast genes themselves depends on the genetic background of the yeast strain.

Acknowledgments

I am greatly indebted to people from the laboratories of Ted Young, Ben Hall, and Mike Smith for providing information and help. During the work I was recipient of a postdoctoral fellowship from the Max Kade Foundation.

[17] J. Bennetzen, and B. D. Hall, *J. Biol. Chem.* **257**, 3026 (1982).

– 70° for 2–4 hr. Lanes 1–4 contain RNAs from cells transformed with pAHG23; lane 5 contains RNA from cells transformed with YEp13. In lanes 1 and 5, strains were grown to a density of 1 to 2 × 10^7 cells/ml; in lanes 2–4, to a density of 5 to 6 × 10^7 cells/ml. Lanes 1 and 2 are hybridized to a probe of rat growth hormone cDNA. The size of the specific mRNAs are 1400 ± 50 bp and 2400 ± 50 bp, respectively. The larger mRNA is present only in cells grown to higher density. Lane 3 shows hybridization to the 1500 bp *ADC1* promoter fragment. Only the large mRNA gives a major positive signal. Lanes 4 and 5 are probed with the 2 μm DNA fragment (bp 105–1998 of 2 μm plasmid) flanking the rGH cDNA on the 3′ end. The RNA hybridization pattern detected in lane 5 is consistent with published results concerning transcription from 2 μm plasmid in yeast.[10]